Research Article

Prokaryotic Expression, Purification, and Polyclonal Antibody Production of a Truncated Recombinant Rabies Virus L Protein

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Background: Rabies virus (RABV) is a deadly neurotropic virus that causes the disease of rabies in humans and animals. L protein is one of the large structural protein of rabies virus, which displays multiple enzymatic activities, and is required for viral transcription and replication.

Objectives: A truncated L protein of Rabies virus is being cloned, expressed and purified to produce relevant polyclonal antibody.

Materials and Methods: The gene fragment of L protein of RABV was subcloned into prokaryotic expression vector pET-28a and transformed into *E. coli* Rosetta DE3 host strain. The recombinant L protein of RABV was expressed and characterized by SDS-PAGE and western blot analysis using anti-his tag antibody. Mice were immunized with the purified recombinant L protein, the reaction of the anti-serum was checked by immunofluorescence and dot-blot, respectively.

Results: The results of PCR and sequencing confirmed that the fragment of L gene of RABV was successfully cloned into the expression vector. The expression of recombinant L protein fragment induced by IPTG was confirmed by the band of 43 kDa in SDS-PAGE and western blot. The antiserum of purified L protein immunized mice was reacted with RABV infected N2a cells and suckling mouse brain tissue lysates.

Conclusions: Our data showed that the recombinant L protein produced by pET-28a vector was very successful, and the purified L protein could efficiently induce the antibody response in mice. The antiserum could recognize the virus in RABV infected cells and tissue very well.

Keywords: L protein; Polyclonal antibody; Prokaryotic expression; Rabies virus

1. Background

Rabies is an old known disease to mankind in recorded history. Approximately 55,000 individuals die from rabies, caused by rabies virus (RABV), worldwide per annum (1). The ~12 kb genome of RABV is composed of five genes that encode the structural proteins of glycoprotein (G), nucleoprotein (N), phosphoprotein (P), matrix protein (M), and large protein (L) (2). The cDNA of L protein encodes a long polypeptide of 2128 amino acids with a relative molecular weight of 243.09 kDa (silver-haired bat-associated strain (SHBRV)). L protein (named after its large molecular weight) is considered to work with the phosphorylated non-catalytic subunit of viral RNA polymerase, P protein, and as a catalytic subunit of RNA polymerase (3). The polymerase complex of L and P displays all the enzymatic activity of transcription, including co-transcriptional modifications of RNAs, such as capping and polyadenylation, and the initiation and elongation of transcripts (4). L protein functions were mostly predicted from studies on vesicular stomatitis virus. In recent years, reports about RABV were mainly focused on the viral G (5-7), P (8-14), M (15-18), and N (9,19-24) proteins. However, the work on L protein of RABV has been relatively limited. Its potential role in the virus cycle and host factors interacting with L protein remains to be determined.

In our previous study (9), we have established 11 mAbs, including three neutralizing antibodies, one anti-nucleoprotein antibody and seven mAbs against phosphoprotein, through a strategy of suckling mouse brain antigen immunization. The large molecular size of L protein and the lack of commercially available antibodies against RABV L protein restricted the progression of RABV L protein research work. Development of an effective antibody is especially

useful for investigating the function of viral proteins and their interaction with host factors.

2. Objectives

L protein plays a key role in the process of RABV infection. The aim of the present study was to clone and express the fragment of L protein of RABV and develop the polyclonal antibody against L protein, in order to provide investigation tools for RABV in the future.

3. Materials and Methods

3.1. Bacterial Strains, Cells, Viruses, Vectors and other Reagents

Top 10 and Rosetta (DE3) *Escherichia coli* strains and pET-28a vector were preserved at our laboratory. RABV HEP-Flury strain was propagated in mouse neuroblastoma N2a cells. The T4 DNA ligase, pMD19-T simple vector, Taq DNA polymerase, restriction enzymes (*BamH* I and *Sal* I), DNA molecular mass markers and PCR product purification kit were purchased from TaKaRa (Dalian, China). PCR product purification kit was purchased from Tiangen Company (Beijing, China). TRIzol reagent was purchased from Invitrogen (CarlsbAd, CA, USA). RevertAid first strand cDNA synthesis kit was product of Thermo Fisher Scientific (Rockford, IL, USA). Ni-NTA agarose bead and Freund's complete adjuvant were purchased from Qiagen (Hilden, Germany) and Sigma-Aldrich (St. Louis, MO), respectively.

3.2. Cloning of L Gene Fragment and Construction of pET28a L (1429-1757) Protein Expression Plasmid

The flury strain of rabies virus L amino acid sequence was downloaded and blasted against NCBI database (http://www.ncbi.nlm.nih.gov/). The sequence was aligned with the L sequence of CVS and ERA strain, common laboratory strains. A part of DNA sequence encoding the L protein, 1429-1757, was picked as suggested by IEDB to be the immunogenic epitope (25).

The *L* gene fragment of RABV was amplified from virus infected neuroblastoma N2a cells by PCR as described by Zhang *et al*. (20). Mouse neuroblastoma N2a cells infected with RABV for 48 h, and the cells were washed with phosphate buffered saline (PBS) two times, and lysed in Trizol. RNA was extracted and reverse transcribed to cDNA with a RevertAid first strand cDNA synthesis kit. cDNA synthesis was performed with 1 µg RNA from N2a cells using oligo(dT) primers. The specific primers for *L* gene fragment 1429-1757 were: forward primer 5′-GCGGATCCTCGATTTGCTTCTTGACA-3′ and reverse primer 5′-GCGTCGACGATATCATCTCCTCCACTCA-3′ (*Bam*H I and *Sal* I restriction sites are underlined). The His-tag was embedded on pET-28a, and expressed as a fusion protein. For pET-28a vector, all constructs were verified by DNA sequencing.

3.3. Expression of the RABV L Protein

E. coli Rosetta was transformed by Recombinant L (1429-1757) protein expression plasmid pET28a-L. A single clone with pET28a-L (1429-1757) was incubated for 20 h in 500 ul LB. The grown bacterial cells were used as seed to inoculate 50 ml LB at 37ºC. At OD_{600} = 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for the induction of recombinant L protein expression and bacterial culture was allowed to grow at 37ºC for 4 h. The bacterial suspension was centrifuged at 10,000 ×g for 30 s and the cell pellet was analyzed on 12% SDS-PAGE. The expressed protein was purified using Ni-NTA agarose bead according to the manufacturer's instructions. The quality of purified recombinant L protein was determined by coomassie-stained SDS-PAGE as well as immunoblotting with anti-His tag monoclonal antibody.

3.4. Production of Polyclonal Antibodies Against Recombinant L (1429-1757) Protein

Mice (three 6-to 8-week-old female BALB/c) were immunized with purified recombinant RABV L (1429-1757) protein mixed with equal volume of Freund's complete adjuvant for the first immunization, and with Freund's incomplete adjuvant for the following two booster immunizations. Each immunization comprised 20-50 µg (per mouse) recombinant RABV L (1429-1757) protein. Sera were collected 14 days after every injection of L protein. The study protocol for the experimental use of the animals was reviewed and approved by the Scientific and Ethics Committee of Kunming University of Science and Technology (P.R. China).

3.5. Immunofluorescence

At 48 h post infection, infected mouse neuroblastoma N2a cells grown on 96-well plate were fixed with ice-cold acetone-methanol (1/1) for 20 min at -20ºC and allowed to be air dried. Primary (mouse anti-L serum, 1:500) and secondary (FITC conjugated goat anti-mouse IgG, 1:200) antibody incubations were carried out by dilution in phosphate-buffered saline containing 0.05% (v/v) tween-20 (PBST) and 5% (w/v) skimmed

milk for 2 h and 1 h at room temperature, respectively. Microscopy was performed with an inverted fluorescence microscope DM IL (Leica, Germany).

3.6. Dot-Blot Assay

Dot-blot was performed as described previously (9), with minor modifications. Briefly, 7.5 µL supernatant of RABV and mock-infected suckling mouse brain tissue lysates in 8 M urea were dotted onto the nitrocellulose membrane. After being air dried, sample-blotted membranes were blocked with 5% (w/v) skimmed milk, and incubated with polyclonal antibody against L protein of RABV, and the goat anti-mouse IgG (H+L)-HRP (KPL, 1:500) . The color reaction was conducted in the presence of tetramethylbenzidine.

4. Results

4.1. RABV L Cloning

Sequence comparison of the L fragment 1429-1757 of RABV L of Flury strain with CVS, ERA and other isolates of rabies showed at least 99% and 96%, respectively. Bepipred Linear Epitope Prediction showed that rabies virus L fragment 1429-1757 had a large number of B-cell epitopes (Figure 1). The cDNA sequence that encodes for amino acid sequence region of 1429-1757 in *RABV L* was PCR amplified (987 bp) from infected N2a cells. The purified products of the amplified target *L* gene fragment and pET-28a vector were double digested by *Bam*H I and *Sal* I. The restricted vector and gene fragment were ligated by T4 DNA ligase. The ligated fragment was transformed into *E. coli* Top10 strain and the positive clone was proved by both bacterial liquid PCR and DNA sequencing.

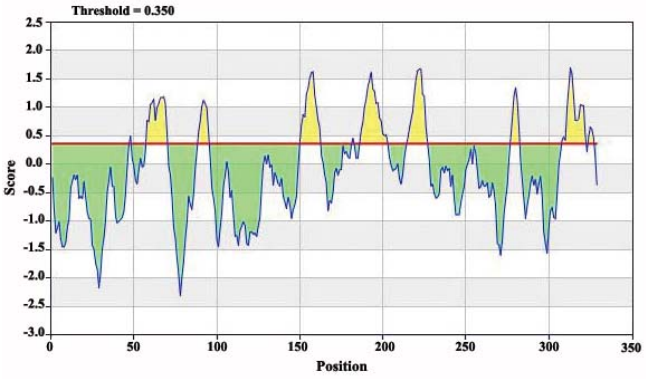

Figure 1. Antigenicity Prediction of Rabies Virus L (1429-1757) gene Fragment from amino acid sequence. The immune epitope database (IEDB) was used to characterize B-cell epitopes of RABV L (1429-1757) Fragment. Bepipred Linear Epitope Prediction (yellow part) showed that rabies virus L fragment 1429-1757 had a large number of B-cell epitopes

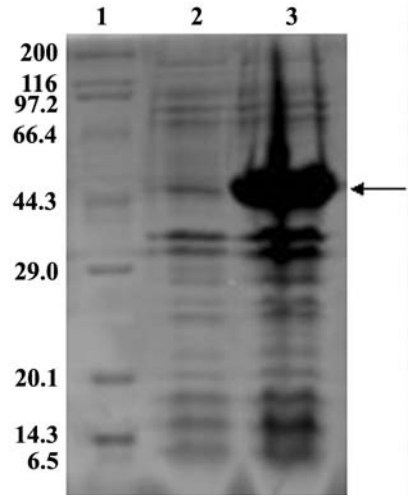

Figure 2. SDS-PAGE analysis of RABV L (1429-1757) expressed in *E. coli.* Lane 1, protein marker; Lane 2, uninduced; Lane 3, 4 h post induction

4.2. Expression and Purification of Recombinant L (1429-1757) Fragment

The expression of L protein in Rosetta strain of *E.coli*, induced by IPTG, was detected (45 kDa) and

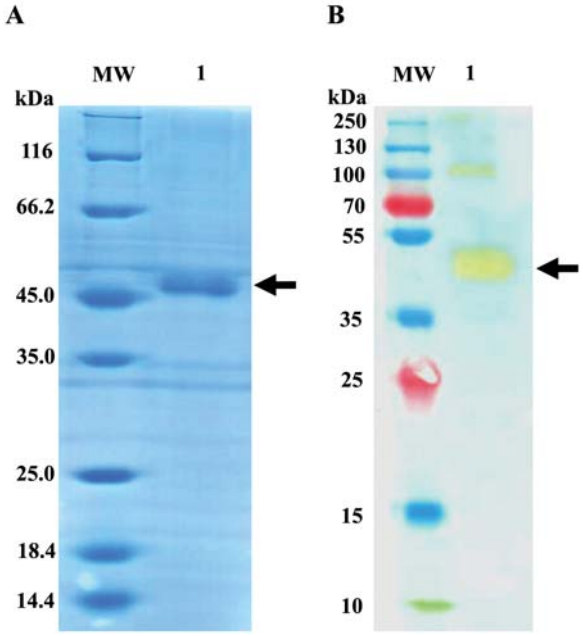

Figure 3. SDS-PAGE and Western blot analysis of the purified recombinant L (1429-1757) protein. (A) Purified protein L (1429-1757). MW, Size marker; Lane 1, purified L protein. (B) Reactivity of recombinant L with anti-His antibody. MW, Size marker; Lane 1, purified L protein

compared with the control bacteria by SDS-PAGE (Figure 2).

The recombinant L protein was extracted from the *E. coli* Rosetta cells by sonication. The lysed inclusion body of L protein was purified on Ni-NTA agarose column (Figure 3A). In Western blots, the recombinant L (1429-1757) protein reacted specifically with the antibody against His tag (Figure 3B), and the band corresponded with the band on SDS-PAGE.

4.3. Antisera Titer Against Recombinant L Protein

After three immunizations, mice antiserum was collected. ELISA demonstrated that the anti-L polyclonal antibody reacted well with the purified L protein. The OD values of different dilutions, collected at different times were shown in (Figure 4). After the 3rd immunization, the titer values were over 0.4 at 1: 16000 dilutions in indirect ELISA. The titer of the antiserum after the 3rd injection, reached 1: 128000 (Figure 5).

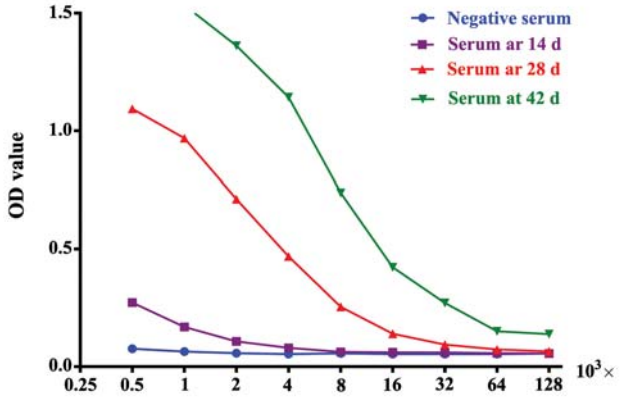

Figure 4. Antiserum titration by ELISA

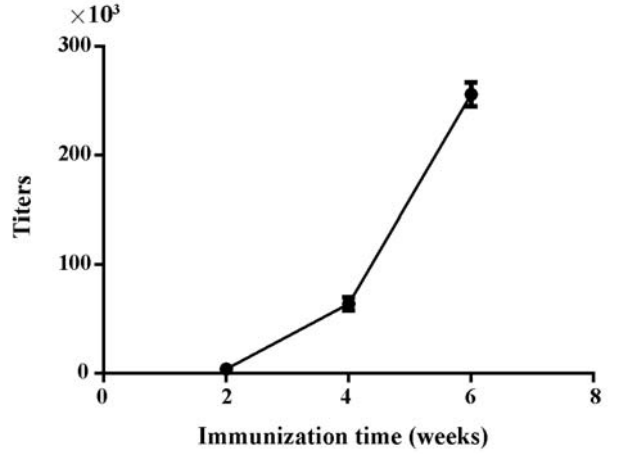

Figure 5. The process of antibody production in mouse antiserum

4.4. Reactivity of Anti-L (1429-1757) Polyclonal Antibodies with RABV Infected N2a Cells and Mouse Brain Tissue Lysates

The reactivity of the anti-L (1429-1757) polyclonal antibody with the native L protein was checked; anti-L (1429-1757) antibody was applied in infected and mock-infected cells of RABV HEP-Flury strain. The polyclonal anti-L (1429-1757) antibody recognized native L protein in RABV-infected N2a cells (Figure 6), with no specific fluorescence in control cells. In dot-blot assay, polyclonal antibody against L protein of RABV strongly reacted with RABV infected suckling mouse brain tissue protein (Figure 7), and only relatively weak reaction was noted with the protein of mock infected mouse brain lysates.

5. Discussion

RABV L protein fragment (1429-1757) was successfully incorporated into the prokaryotic vector pET-28a to produce the recombinant L protein, followed by

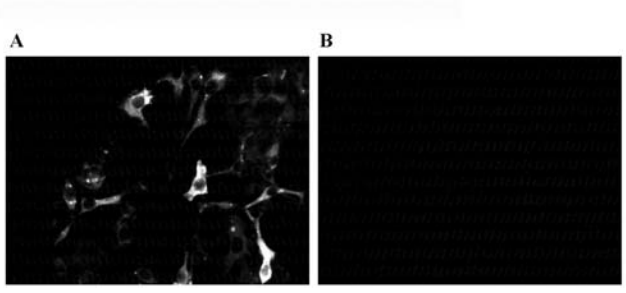

Figure 6. Reactivity of anti-L serum with RABV infected neuroblastoma N2a cells. A: RABV infected neuroblastoma N2a cells at 48 h post infection. B: Mock-infected neuroblastoma N2a cells at 48 h post infection

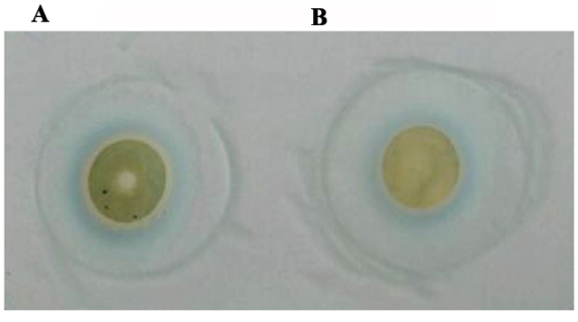

Figure 7. Reactivity of anti-L serum with RABV infected suckling mouse brain tissue. A: RABV infected suckling mouse brain tissue supernatant. B: Mock-infected suckling mouse brain tissue supernatant

the production of its polyclonal antibody. It has been previously accepted that the L protein is the largest protein of RABV, and has multifunctional activities including catalytic activities involved in genomic RNA synthesis, viral transcription and phosphorylation of viral phosphoprotein (3). A reverse genetics approach has been applied to make intracellular reconstitution of the transcriptionally active RABV ribonucleoprotein particles from plasmid encoded proteins that addressed the role of conserved L protein sequences of RABV involved in RNA polymerase activity (26). Due to its large protein size, it would be very hard to express such a big protein in prokaryotic or eukaryotic system, which restricted the development of its antibody and research progress of L protein. By far amongst rabies virus proteins, G protein (27-31), nucleoprotein (32-33), M protein (34) and phosphoprotein (35) have been successfully expressed in varieties of hosts and most often led to production of an antibody. However and until now, no commercial antibody for RABV L protein was developed to be introduced to the market. Previously, we have developed a series of monoclonal antibodies against structural proteins of rabies virus through the immunization of rabies suckling mouse brain antigen, including nucleoprotein, phosphoprotein and neutralizing antibodies (9). However, we have never obtained any monoclonal antibodies against L protein of rabies virus, most likely because L protein is a nonstructural protein and its expression level was too low to induce sufficient antibody response. Or it's caused by the uncertainty in the process of screening and subcloning of mAb cell lines, which made we lose the chance to get an anti-L hybridoma cell line (9).

The functions and characteristics of the large polymerase protein (L) of RABV are mainly predicted from the prototypic rhabdovirus, vesicular stomatitis virus, and therefore a thorough functional analysis of this protein seems inevitable. In order to solve some unanswered questions about the function of L protein, we took one of the first steps towards its characterization by developing a specific antibody.

Some *in silico* analysis was performed to identify the conserved sequence (1429-1757) of L protein, and the distribution of linear B cell epitopes (data not shown). Accordingly, protein L fragment of 1429-1757 was considered as a proper immunogenic region for antibody production. Attempt towards its expression in BL21 (DE3) was failed, but Rosetta (DE3) could produce the recombinant L protein. This demonstrated that the codon usage bias of L protein might resulted in the failure of L protein expression in *E. coli* BL21, and switching of expression systems to Rosetta (DE3) corrected for the codon bias.

Although the polyclonal antibody against the L protein of RABV could recognize the RABV infected suckling mouse brain tissue lysates very well, the mock infected mouse brain tissue showed some color reactions as well. This cross-reactivity might be due to the lower specificity of polyclonal in comparison to monoclonal antibody (36) or as a result of the complexity of mouse brain tissue. Having said this, the intensity of RABV infected mouse brain samples demonstrated to be stronger than the mock-infected samples. It showed that the polyclonal antibody reacted with the L protein of RABV infected mouse brain.
In summary, RABV L protein fragment (1429-1757) was cloned and expressed in *E. coli* Rosetta strain. The recombinant protein of L was purified by Ni-NTA agarose column and characterized using anti-His antibody in RABV infected mouse neuroblastoma N2a cells. These purified recombinant L protein and polyclonal antibody are potentially useful tools for the virus diagnostics and novel therapeutic target identification, and would be helpful to further characterize the RNA polymerase of RABV.

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